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Original Article

Antioxidant and antimicrobial activities of *Salix babylonica* extracts

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ABSTRACT

The methanolic extracts of the leaves and bark of *Salix babylonica* and their petroleum ether, methylene chloride and ethyl acetate fractions were investigated for their antioxidant and antimicrobial activities. They were also quantitatively assayed for their phenolic contents. Antimicrobial activities were determined against a panel of microorganisms; the Gram-positive bacteria (*Staphylococcus aureus*), the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and the yeast-like pathogenic fungus *Candida albicans.* Antioxidant activity was carried out using ABTS antioxidant assay and phosphomolybdenum antioxidant assay. Total phenolic content was carried out using Folin-Ciocalteu method. The ethyl acetate fraction of the methanolic extract of the bark showed the highest total phenolic content, it showed also the highest antioxidant activity. The methylene chloride fraction of the bark showed the strongest antibacterial activity against *E. coli*. The ethyl acetate fraction of the methanolic extract of the bark showed strong antibacterial activity against *S. aureus*. The petroleum ether, methylene chloride and ethyl acetate fractions of the methanolic extract of the bark exhibited moderate antifungal activities against *Candida albicans*.

Keywords: ABTS antioxidant assay, phosphomolybdenum antioxidant assay, *E. coli*, Phenolic content.

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INTRODUCTION

Salix babylonica L., also called Weeping Willow, belongs to family Salicaceae. It is a broad-headed large tree with flexile hanging greenish to brown branches [1]. It is widely cultivated in Asia, Europe and America [2]. In the traditional Chinese medicine, *S. babylonica* is used in the treatment of hepatitis with jaundice; chronic pelvic inflammation; aplastic anemia and rheumatoid arthritis [3, 4, 5, 6]. Externally, *S. babylonica* is used in topical preparations for treating *Tinea manuum*, *Tinea pedis* and onychomycosis [7]. It is also used in analgesic plasters and ointments for relieving pain of cancer patients and treating skin ulcer [8, 9]. The Assyrians and ancient Egyptians used the leaves and the bark in cases of musculoskeletal pain. Plants belonging to genus *Salix* are famous for their contents of salicin, the precursor of acetylsalicylic acid (aspirin), besides flavonoids, terpenoids, lignans and phenolic compounds. These compounds possess versatile biological activities as being analgesic, antipyretic and anti-inflammatory [10, 11]. Phenolic compounds are considered effective antioxidants owing to their ability to neutralize the free radicals that cause oxidative stress. Many studies reported that the phenolic compounds possess antimicrobial activity [12]. In this study, the antioxidant and antimicrobial activities of the methanolic extracts of *Salix babylonica* leaves and bark and their petroleum ether, methylene chloride and ethyl acetate fractions are evaluated. Moreover, the total phenolic contents of these methanolic extracts and their fractions are quantitatively estimated.

MATERIALS AND METHODS

General:

Equipments: Rotary flash evaporator (Büchi, Switzerland) for evaporation of the extracts and fractions, Spekol 11 (Carl Zeiss-Jena) spectrophotometer for measuring the absorbance in total phenolic content assay and ELx800UV (USA) for measuring the absorbance in ABTS and phosphomolybdenum assay.

Reagents: Folin Ciocalteu reagent, ABTS (2,2'Azino-bis-(3-ethyl benzthiazoline-6-sulfonic acid) reagent and Phosphomolybdenum reagent [28 mM sodium phosphate (100 ml) and 4 mM ammonium molybdate (100 ml) in 0.6 M sulphuric acid (100 ml)].

Media: Mueller Hinton agar medium for the antibacterial assay. YPD (Yeast extract- Peptone - Dextrose) agar medium for the antifungal assay.

Microorganisms: The Gram-positive bacteria (*Staphylococcus aureus*), the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), and the yeast-like pathogenic fungus *Candida albicans*, these microorganisms were provided from the laboratory of Microbiology and Immunology department, Faculty of Pharmacy, Mansoura University, Egypt.

Plant material: The leaves and bark of *S. babylonica* L. were collected from the trees growing on the Nile River in Mansoura, Egypt in June 2014 and in February 2015 respectively. The plant was authenticated by Prof. Mohamed Farid Lahloub, Professor of pharmacognosy, Department of pharmacognosy, Faculty of pharmacy, Mansoura University, Egypt.

Preparation of the extracts: *For the leaves*: The powdered, air dried leaves (4 Kg) were extracted by maceration in methanol at room temperature (9 x 6 L). The collected methanolic extract was evaporated under reduced pressure to give dark green viscous residue (1069 g). The dried methanolic extract was dissolved in the least amount of methanol, diluted with 1000 ml distilled water, fractionated using solvents of increasing polarities; petroleum ether (11 x 1 L), methylene chloride (4 x 1 L), ethyl acetate (7 x 1 L) and *n*-butanol (1 x 1 L). The solvent, in each case, was evaporated to dryness under reduced pressure giving petroleum ether fraction (121.6 g), methylene chloride fraction (33.9 g), ethyl acetate fraction (97.7 g) and n-butanol fraction (40 g).

For the bark: The powdered, air dried bark (2.5 Kg) were extracted by maceration in methanol at room temperature (6 x 2.5 L). The collected methanolic extract was evaporated under reduced pressure giving (400 g) of dark brown residue. The dried methanolic extract was dissolved in the least amount of methanol, diluted with 1000 ml distilled water, fractionated using solvents of increasing polarities; petroleum ether (8 x 3 L), methylene chloride (6 x 2 L) and ethyl acetate (5 x 3.5 L). The solvent, in each case, was evaporated to dryness under reduced pressure giving petroleum ether fraction (60.6 g), methylene chloride fraction (40.4 g) and ethyl acetate fraction (205 g).

Total phenolic content: The content of total phenolic compounds was evaluated according to Folin-Ciocalteu method [13]. From each extract or fraction 0.1g was dissolved in 1 ml methanol. Then, 2.8 ml distilled water, 2.0 ml of 2% (w/v) sodium carbonate and 0.1 ml of 50% (v/v) of Folin–Ciocalteu reagent were added to the aliquots of 0.1 ml from the previously prepared extracts and fractions solutions. The previous mixture was kept for 30 minutes at room temperature 25^{o} C. The absorbance of the resulting color was measured at 750 nm against methanol as blank. A standard curve of gallic acid was previously prepared by the

same manner for the quantitative estimation of phenolic content. Total phenolic contents are expressed as microgram gallic acid equivalent (GAE)/ 100 mg of sample based on dry weight.

Antioxidant activity: Antioxidant power was quantitatively evaluated using ABTS antioxidant assay [14] and phosphomolybdenum antioxidant assay [15].

ABTS antioxidant assay: The $ABTS^+$ radical cation solution was prepared as reported in [14] The absorbance ($A_{control}$) of the green-blue solution ($ABTS^+$ radical solution) was recorded at λ_{max} 734 nm. The absorbance (A_{test}) was measured upon the addition of 20 µl of 1 mg/ml solution of the test sample in spectroscopic grade MeOH/phosphate buffer (1:1 v/v) to the $ABTS^+$ radical solution. Ascorbic acid 20 µl (2 mM) solution was used as standard antioxidant (positive control). Blank experiment was run using solvent without ABTS.

Phosphomolybdenum antioxidant assay: In capped test tubes, the reaction mixture was prepared by adding 0.3 mL of a 1 mg/mL extract or fraction solution in methanol with 2.7 mL phosphomolybdenum reagent. The previous mixture was incubated for 90 min in a water bath at 95 °C then cooled to room temperature. The absorbance of the solution was measured using a UV-visible spectrophotometer at 750 nm against a blank (0.3 mL methanol without plant extract). The antioxidant capacity was reported as µg of ascorbic acid equivalents (AAE)/ mL.

Antimicrobial activity: The antimicrobial activity was evaluated against a panel of sensitive microorganisms including the Gram-positive bacteria (*Staphylococcus aureus*), the Gram-negative bacteria (*Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), and the yeast-like pathogenic fungus *Candida albicans* as described previously[16]. The primary screening was carried out by the agar disc-diffusion method. The overnight cultures of the different strains were diluted to optical density OD_{600nm} of 0.1 (equivalent to 8 $x10^7$ cells/ml), except for *C. albicans* that was diluted to OD_{600nm} of 0.5 (equivalent to 8 $x10^7$ cells/ml). The diluted cultures were spread using sterile swab on the surface of Mueller Hinton agar plates in case of screening the antibacterial activity and on YPD agar plates in case of antifungal activity. The plates were left to dry, then, followed by the application of sterile cellulose disc papers (6 mm each) on the surface of the media on appropriate distances from each other. The extracts and fractions were dissolved in DMSO to be used as stock solutions with a concentration of 10 mg/300 µl. From each stock solution, 3 µl were loaded into the disc papers, thus each disc paper is loaded with 100 µg. Ampicillin (100 µg/disc) and gentamicin (100 µg/disc) were used as standard antibacterial compounds in the antibacterial assay, while fluconazole (40 µg/disc) was used as standard in the antifungal assay. The negative control used was 100% DMSO. The plates were incubated at 37°C for 24 h. The diameter of the growth inhibition zone was determined to the nearest millimeter using a caliber.

RESULTS AND DISCUSSION

This work is concerned with the evaluation of the total phenolic contents; antioxidant and antimicrobial activities of the methanolic extracts of the leaves and bark of *Salix babylonica* and their fractions.

Total phenolic content: Quantitative analysis by UV-visible spectrophotometer after establishment of standard curves with Gallic acid (table 1) revealed that the methanolic extracts of both the leaves and bark of *Salix babylonica* possess moderate phenolic contents. Studying the results of the fractions revealed that the ethyl acetate fractions of both the leaves and bark possessed the highest total phenolic contents; 35.063 and 59.788, respectively, followed by the methylene chloride fractions; 27.492 and 21.270, respectively. Petroleum ether fractions possessed the lowest phenolic contents; 6.133 and 6.741, respectively. The ethyl acetate fraction of the methanolic extract of the bark possessed the highest phenolic content amongst all the tested fractions.

Antioxidant activity: Polyphenolic compounds in plants are responsible for their antioxidant activity, which have other pro-health activities, such as antimutagenic, anticarcinogenic, and anti-aging. Polyphenols have the ability to scavenge reactive oxygen species, preserving the genomic stability of cells through elimination of carcinogens and interference with DNA adduct formation [17]. Therefore, the quantitative evaluation of the *in-vitro* antioxidant activity is important.

The antioxidant activities of the methanolic extracts of the leaves and the bark of *Salix babylonica* and their fractions were evaluated using both ABTS method and phosphomolybdenum method. Results are showed in tables 2 and 3. The results of ABTS assay (table 2) revealed that the methanolic extract of the bark exhibited a potent antioxidant activity nearly equal to that of ascorbic acid. The ethyl acetate and methylene chloride fractions of the methanolic extract of the bark showed potent antioxidant activities compared to that of ascorbic acid (87.4 and 86.5), while

petroleum ether fraction showed weak antioxidant activity. The methanolic extract of the leaves showed high antioxidant activity compared to that of ascorbic acid but still less than that showed by the methanolic extract of the bark. Its methylene chloride fraction showed a potent antioxidant activity (86.9, table 2), while the ethyl acetate and petroleum ether fractions showed low antioxidant activities.

The results of antioxidant activity revealed by phosphomolybdenum antioxidant assay method (table 3) were consistent with those revealed by ABTS method. Amongst all tested fractions, both methylene chloride and ethyl acetate fractions of the bark possess the highest antioxidant activities. This may be attributed to their high phenolic content. The anti-oxidant activity of the different extracts is mainly attributed to their phenolic content.

Antimicrobial activity: The antimicrobial activity was carried out against a panel of microorganisms; Gram-positive bacteria (*S. aureus*), the Gram-negative bacteria (*E. coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*), and the yeast-like pathogenic fungus *Candida albicans.* Results are shown in table 4.

Results showed that both methanolic extract of the leaves and the bark exhibited moderate or weak antimicrobial activities against all tested microorganisms. The methylene chloride fraction of the bark showed strong antibacterial activity against *E. coli*. Both petroleum ether and ethyl acetate fractions of the leaves and bark showed moderate antibacterial activities against *E. coli.*

All tested extracts and their fractions showed weak antibacterial activities against *K. pneumoniae*, except butanol fraction of the leaves, petroleum ether and methylene chloride fractions of the bark that showed no antibacterial activities.

Methanolic extract of the leaves and its ethyl acetate fraction showed moderate antibacterial activity against *Pseudomonas aeruginosa.* Methanolic extract of the bark and its petroleum ether fraction showed moderate antibacterial activity against *Pseudomonas aeruginosa*.

Ethyl acetate fraction of the bark exhibited strong antibacterial activity against *S. aureus*, while petroleum ether fraction of the bark showed moderate activity. Other fractions showed weak activities.

Although methanolic extract of the bark showed weak antifungal activity against *Candida albicans*, its petroleum ether, methylene chloride and ethyl acetate fractions showed moderate antifungal activity where they showed inhibition zones of (14, 10 and 10 mm, respectively) as shown in table 4.

CONCLUSION

It could be concluded that the leaves and bark of *Salix babylonica* contain considerable amounts of phenolic compounds that is reflected in their potent antioxidant activities. The plant has moderate antimicrobial activity. These results provide scientific evidence to explain its abundant uses in traditional medicine and make it possible to classify it among the antioxidant plants.

Conflict of interest: There is no conflict of interest.

Table (1): The total polyphenolic content of the methanolic extracts of the leaves and bark of *Salix babylonica* and their fractions

No.	Extracts & fractions	µg/100 mg (GAE) Gallic acid equivalent
1.	Methanolic extract of the leaves	27.217
2.	Pet. ether fraction of the leaves	6.133
3.	Methylene chloride fraction of the leaves	27.492
4.	Ethyl acetate fraction of the leaves	35.063
5.	Butanol fraction of the leaves	12.434
6.	Methanolic extract of the bark	21.341
7.	Pet. ether fraction of the bark	6.741
8.	Methylene chloride fraction of the bark	21.270
9.	Ethyl acetate fraction of the bark	59.788

Table (2): Results of antioxidant activity screening of the methanolic extracts of the leaves and bark of *Salix babylonica* and their fractions using ABTS anti-oxidant assay:

No.	Extracts & fractions	% inhibition
	Blank	0
	Ascorbic-acid (standard)	89.0%
1.	Methanolic extract of the leaves	65.4%
2.	Pet. ether fraction of the leaves	26.4%
3.	Methylene chloride fraction of the leaves	86.9%
4.	Ethyl acetate fraction of the leaves	28.5%
5.	Butanol fraction of the leaves	59.9%
6.	Methanolic extract of the bark	87.3%
7.	Pet. ether fraction of the bark	14.2%
8.	Methylene chloride fraction of the bark	86.5%
9.	Ethyl acetate fraction of the bark	87.4%

$$\% \text{ inhibition} = [(A_{control} - A_{test}) / A_{control}] \times 100$$

Table (3): Results of antioxidant activity screening of the methanolic extracts of the leaves and bark of *Salix babylonica* and their fractions using phosphomolybdenum antioxidant assay:

No.	Extracts & fractions	TAC (Total Antioxidant Capacity)
	Blank	0
1.	Methanolic extract of the leaves	175.35
2.	Pet. ether fraction of the leaves	218.35
3.	Methylene chloride fraction of the leaves	242.35
4.	Ethyl acetate fraction of the leaves	90.85
5.	Butanol fraction of the leaves	84.35
6.	Methanolic extract of the bark	304.35
7.	Pet. ether fraction of the bark	279.35
8.	Methylene chloride fraction of the bark	414.34
9.	Ethyl acetate fraction of the bark	488.85

Table (4): Results of antimicrobial activity screening of the methanolic extracts of the leaves and bark of *Salix babylonica* and their fractions:

No.	Extracts&fractions	Inhibition Zone diameter (mm)				
		E. coli	***K. pneumoniae***	***P. aeruginosa***	***S. aureus***	***C. albicans***
	Ampcillin	25	18.8	18.1	21	-
	Gentamicin	26	25	20.4	31	-
	Fluconazole	-	-	-	-	24.7
	DMSO	-	-	-	-	-
1.	Methanolic extract of the leaves	8	9	10	8	7
2.	Pet. ether fraction of the leaves	10	9	8	7	7
3.	Methylene chloride fraction of the leaves	9	7	9	-	7
4.	Ethyl acetate fraction of the leaves	10	8	12	8	7
5.	Butanol fraction of the leaves	-	-	-	-	-
6.	Methanolic extract of the bark	8	9	10	8	7
7.	Pet. ether fraction of the bark	10	-	11	11	14
8.	Methylene chloride fraction of the bark	16	-	8	9	10
9.	Ethyl acetate fraction of the bark	10	7	9	16	10

Not active (-); weak activity (2-9 mm); moderate activity (10-15 mm); strong activity (> 15 mm). *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. aureus*: *Staphylococcus aureus* and *C. albicans*: *Candida albicans.*

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